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FURTHER STUDIES ON CYSTATHIONINE SYNTHETASE-SERINE DEAMINASE OF RAT LIVER

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SUMMARY

1. The purity of rat liver cystathionine synthetase-serine deaminase was more than doubled over that previously reported through the use of zone electrophoresis. The ratio of the two enzymic activities of the more purified preparation remained unchanged.

2. Enzymic activity was slightly inhibited by certain pyridoxine derivatives and strongly inhibited by carbonyl reagents, sulfhydryl reagents, various metal chelating agents, and by a number of heavy metal ions. Mg^{++} and Mn^{++} slightly stimulated the serine deaminase activity.

3. Study of the substrate specificity of the enzyme showed that only L-serine and L-threonine were deaminated and only homocysteine was utilized for synthesis of a sulfur-containing diamino acid with L-serine. L-cysteine strongly inhibited cystathionine synthesis.

INTRODUCTION

A procedure for the partial purification of cystathionine synthetase-serine deaminase from rat liver and some of the properties of this enzyme were reported earlier[1]. The

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present communication deals with the further purification of the enzyme by column electrophoresis, studies on the substrate specificity of its dual enzyme activities, and inhibition and activation by a variety of reagents..

EXPERIMENTAL MATERIALS AND METHODS

Materials

Geon (type 426) resin for use in column electrophoresis was generously supplied by the B. F. Goodrich Chemical Co., Avon Lake, Ohio. It was washed before use and the column was packed in the manner described by PORATH[2].

The chemical compounds employed in this work and their sources are the same as previously reported[1].

Enzyme preparation

Preliminary purification of the enzyme for column electrophoresis was carried out by the published method[1]. This preparation was stored with ammonium sulfate as a frozen thick paste with almost no loss of activity. The serine deaminase specific activity of the preparations varied between 150 and 160.

Analytical methods

Protein concentrations were determined by the method of MOKRASCH AND MCGILVERY[3], based on the biuret reaction.

The enzyme assay methods previously reported[1] were employed, with the exception that mercaptoethanol was substituted for dimercaptopropanol because the former is more stable and more soluble in water, and, also, because at the concentration employed ($1.3 \cdot 10^{-4}$ *M*) mercaptoethanol slightly activated while the dimercaptopropanol somewhat inhibited the serine deaminase reaction.

In the assays, 0.25 mmoles of DL-homocysteine or 0.2 mmoles of L-serine/3 ml of incubation medium was employed as the substrates. The reaction medium contained between 0.35 to 0.45 mg of enzyme protein; 0.1 *M* potassium borate buffer, pH 8.3, and $5 \cdot 10^{-5}$ *M* pyridoxal phosphate with other components as indicated in the respective tables. The incubations were run for 30 min at 37° in a Dubnoff shaking incubator.

For column electrophoresis, the packed column (2 × 55 cm) was washed and equilibrated with 0.05 *M* potassium phosphate buffer, pH 7.5 for 24 h. The frozen enzyme preparation was dissolved in 6 volumes of 0.05 *M* potassium phosphate buffer, pH 7.5, containing $5 \cdot 10^{-5}$ *M* EDTA (ethylenediaminetetraacetic acid), and then dialyzed for 12 h against the same buffer. The small amount of protein precipitate that formed was removed by centrifugation.

The clear, yellowish solution (4 ml containing about 40 mg of protein) was slowly pipetted into the column. It was further allowed to travel, as observed by the movement of the yellow color, to a point about 17 cm from the top of the column by the gradual addition of the phosphate buffer. After setting the column into the electrophoresis apparatus, the two limbs of the assembly were dipped into the electrode vessels containing the same buffer. The power supply was adjusted to 750 V, allowing approximately 50 mA of current to flow for 16 h. At the end of the run, the column was removed, a stopcock was attached and it was placed over a fraction collector

and the contents were eluted with the same phosphate buffer at a flow rate of approx. 18 to 20 ml/h. 25 fractions were collected of about 3 ml each. The whole operation was carried out at about 6°.

The protein content and serine deaminase activity of the fractions were then determined, and the results plotted (Fig. 1). It was observed that the highest enzymic activity was always found in the fractions which were the deepest yellow in color, which aided in their location. This color was found to travel together with the enzyme protein. The L-serine deaminase purified in this manner attained a specific activity of 346 units/mg of protein.

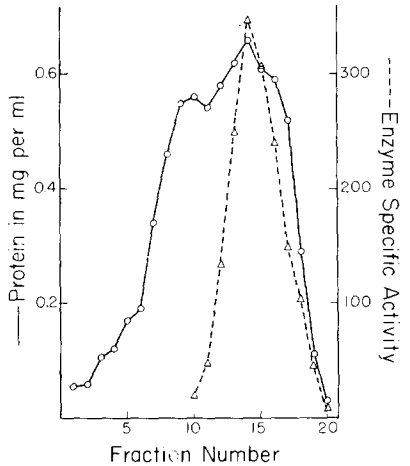

Fig. 1. Partition of protein and enzyme activity of cystathionine synthetase-serine deaminase on column electrophoresis.

In a second run performed in exactly the same way, a specific activity of 334 serine deaminase units/mg of protein was obtained. The specific activity of cystathionine synthetase, determined on the fraction with the highest serine deaminase activity, was 121. This gives a ratio for serine deaminase to cystathionine synthetase activities of 2.76, which is in good agreement with the previous findings[1].

For the additional studies reported here, batches of 100 to 120 mg of enzyme protein were fractionated by electrophoresis and those of highest enzyme activity collected. The combined fractions were brought to 50% saturation with ammonium sulfate by the slow addition of solid salt while stirring the mixture mechanically and periodically adjusting the pH to 7.2 with ammonium hydroxide. After standing for 6 h at 0°, the mixture was centrifuged at 8,000 $\times$ g and the resulting precipitate stored at —20°.

Effect of various inhibitors on the serine deaminase activity of the enzyme

Effect of pyridoxine derivatives: Addition of pyridoxal phosphate to the incubation media appreciably increased the enzyme activity[1]. It was of interest therefore to determine the effect of pyridoxine derivatives on the enzyme activity. Tests were made with 4-deoxypyridoxine-5-phosphate, 4-deoxypyridoxine hydrochloride, and pyridoxine hydrochloride at a concentration of $4 \cdot 10^{-3} M$ on serine deaminase activity in the presence of $5 \cdot 10^{-5} M$ pyridoxal phosphate. The inhibitory effect found was small, amounting to only 10 to 15 %.

Inhibition by carbonyl reagents: The effect of a number of carbonyl reagents on serine deaminase activity are summarized in Table I. In this experiment, a second control was also run in the same manner as for the enzyme assay, except that the carbonyl reagent was added after the incubation, followed by the addition of trichloroacetic acid. A correction for any action of the carbonyl reagent on the development of the color of the pyruvyl-2,4-dinitrophenylhydrazone was thus determined and applied to the enzyme results. All carbonyl reagents inhibited the enzyme activity, decreasing in effectiveness in the order; hydroxylamine > cyanide > isonicotinic acid hydrazide > sodium bisulfite.

TABLE I

INHIBITION OF SERINE DEAMINASE BY CARBONYL REAGENTS

Components not listed in text were enzyme, 0.45 mg, EDTA, $5 \cdot 10^{-4}$ *M*; mercaptoethanol, $1.3 \cdot 10^{-4}$ *M*. Concentration of inhibitors, $6.7 \cdot 10^{-3}$ *M*.

Inhibitor	*Enzyme activity μmoles serine deaminated*	*Inhibition per cent*
None	65	
NaCN	5.6	91
Hydroxylamine hydrochloride	4	94
Isonicotinic acid hydrazide	44	32
$NaHSO_3$	52.2	20

TABLE II

EFFECTS OF METAL IONS ON SERINE DEAMINASE

Enzyme protein 0.36 mg. Concentration of each metal ion, $3.3 \cdot 10^{-4}$ *M*.

Metal ions added	*Enzyme activity μmoles serine deaminated*	*Per cent change*
None	52.3	
Cu^{++}	4.2	— 92
Hg^{++}	1	— 98
Cd^{++}	1.6	— 97
Fe^{++}	47.7	— 13
Co^{++}	51	— 7
Ni^{++}	48.8	— 12
Mn^{++}	54.6	+ 4
Zn^{++}	53	+ 1
Mg^{++}	57	+ 9

Action of sulfhydryl reagents: Determination of the effect of *p*-chloromercuribenzoate, N-ethylmaleimide, *o*-iodosobenzoate and iodoacetate on serine deaminase activity at approximately 10^{-3} *M*, yielded the following result. The first three strongly inhibited the enzymic activity (80–100 %); iodoacetate inhibited only moderately (37 %).

Inhibition by metal ions: Table II summarizes the effect of various metal ions on serine deaminase activity. Controls which differed from the enzyme assay only in that the enzyme preparation was added after incubation were run with each assay. With the exception of Mg^{++}, Mn^{++} and Zn^{++}, all of the metal ions tested inhibited the enzymic activity. The most effective inhibitors were Hg^{++}, Cd^{++} and Cu^{++} which

produced almost complete inhibition at the concentration employed. Mg^{++} and Mn^{++} caused a small but significant activation of the serine deamination reaction.

Effect of metal chelating and related compounds: It was of interest to determine the effect of varying concentrations of EDTA, mercaptoethanol and dimercaptopropanol on serine deaminase activity in the presence of pyridoxal phosphate because of their use as components of the incubation media. Both EDTA and mercaptoethanol stimulated the enzymic activity slightly at the molar concentrations of $5 \cdot 10^{-4}$ and $1.3 \cdot 10^{-4}$ respectively. At higher concentrations both substances become inhibitory. The inhibition increases gradually with increasing concentration of the reagent, and becomes of considerable magnitude at a concentration of 10^{-2} *M* (—18.5 % for EDTA and —11.5 % for mercaptoethanol).

Dimercaptopropanol, on the other hand, was found to inhibit the enzymic activity even at the lowest concentration tested ($1.3 \cdot 10^{-4}$ *M*), and the degree of inhibition increased greatly at higher concentrations.

The effects of a number of heterocyclic aromatic compounds on serine deaminase activity are shown in Table III. Of the reagents tested, 8-hydroxyquinoline and *o*-phenanthroline showed a low degree of inhibition at the concentration of $4 \cdot 10^{-3}$ *M*. The most effective inhibitor in this group was 8-hydroxyquinoline-5-sulfonic acid, (30 % inhibition). On the other hand, 2-hydroxyquinoline produced no inhibition possibly because this reagent does not form metal chelates[4].

Unlike the other metal chelating agents tested, 2,2′-bipyridine appreciably stimulated the enzymic reaction both in the absence and presence of EDTA. This may be attributed to chelation by this reagent of harmful metal ions such as those of iron or copper, thereby inactivating them.

TABLE III

EFFECT OF CHELATING AGENTS ON SERINE DEAMINASE ACTIVITY

Enzyme protein 0.37 mg; EDTA, $5 \cdot 10^{-4}$. Concentration of chelating agents, $4 \cdot 10^{-3}$ *M*.

Compound added	*Enzyme activity μmoles serine deaminated*	*Per cent change*
None	53	
8-hydroxyquinoline	46.2	— 13
o-phenanthroline	50	— 6
2-hydroxyquinoline	53	0
8-hydroxyquinoline-5-sulfonic acid	37.2	— 30
2,2′-bipyridine	62.2	+ 17

Substrate specificity of serine deaminase-cystathionine synthetase

The relative activities of the enzyme on a number of hydroxy and S-containing amino acids and alanine are summarized in Table IV. The enzyme action on the amino acids was determined by the formation of α-keto acid. Lithium pyruvate was used as the color standard, regardless of the nature of the α-keto acid expected to be produced from a given amino acid.

Of the hydroxyamino acids tested, L-threonine was deaminated at about 20 % of the rate of L-serine. Possibly this activity may reflect contamination of the enzyme with L-threonine deaminase, also present in rat liver, but this cannot be accepted

TABLE IV

ACTIVITY OF SERINE DEAMINASE ON HYDROXY AND SULFUR CONTAINING AMINO ACIDS

Enzyme protein, 0.36 mg; EDTA, $5 \cdot 10^{-4} M$; mercaptoethanol, $1.3 \cdot 10^{-4} M$.

Amino acid	*Amount added µmoles*	*Enzyme activity µmoles serine deaminated*
L-serine	200	57
L-threonine	200	11.4
L-homoserine	200	0.6
β-hydroxy-DL-aspartic acid*	100	0
D-glucosamine hydrochloride	200	1.1
DL-homocysteine	250	0.5
L-cysteine	200	0
DL (+ allo)-cystathionine	50	0
Lanthionine (L and meso)	50	0.6
S-isopropyl-DL-homocysteine	50	0
S-methyl-L-cysteine	100	0.2
S-carboxylmethyl-L-cysteine	50	0.6
DL-ethionine	100	0.4
L-methionine	100	1.0

* A synthetic preparation kindly furnished by Dr. E. KUN. Probably composed of mixture of the several possible configurations.

with certainty. The other hydroxyamino acids, and also the sulfur-containing amino acids tested were found to be essentially inert as substrates for the enzyme.

Activity of enzyme on L-*serine in presence of certain sulfur-containing amino acids.*

The effect of certain sulfur-containing amino acids on the serine deaminase and apparent cystathionine synthetase activity of the enzyme is recorded in Table V. The synthetase activity of the enzyme was evaluated by determining the amount of L-serine which reacted but was not converted to pyruvic acid, as described in the previous communication[1].

The results obtained with homocysteine and cysteine parallel those already published. Both of these amino acids virtually eliminate serine deaminase activity, but only homocysteine induces the synthetase reaction.

Substitution of the sulfhydryl group of these amino acids greatly reduced the

TABLE V

ACTIVITY OF SERINE DEAMINASE AND CYSTATHIONINE SYNTHETASE ON SULFUR CONTAINING AMINO ACIDS IN PRESENCE OF L-SERINE

Enzyme protein, 0.36 mg: mercaptoethanol, $1.3 \cdot 10^{-4}$; EDTA, $5 \cdot 10^{-4} M$; 200 µmoles of L-serine and the sulfur amino acid in the amounts shown below.

Sulfur amino acid	*Amount added µmoles*	*Serine deaminase activity µmoles serine utilized*	*Cystathionine synthetase activity µmoles serine utilized*
None		58	
DL-homocysteine	250	1.3	21.5
L-cysteine	200	1.1	0
S-isopropyl-DL-homocysteine	100	45.7	3.3
S-methyl-L-cysteine	100	35	2.4

degree of inhibition of serine deaminase activity. Both *S*-isopropyl-DL-homocysteine and *S*-methyl-L-cysteine caused some apparent synthetase activity. For the former, this might be explained by some contamination or decomposition resulting in the occurrence of homocysteine. The explanation of the result indicating a synthetic effect of *S*-methyl-L-cysteine is not readily apparent.

Activity of the enzyme on hydroxyamino acids in the presence of homocysteine

The relative synthetase activity of the enzyme was tested on a number of hydroxyamino acids, in the presence of DL-homocysteine (Table VI). Synthetic activity was estimated by the disappearance of homocysteine from the reaction mixtures by means of the nitroprusside method of GRUNERT AND PHILIP[5]. Two controls were run simultaneously with the enzyme assay; in one, the enzyme preparation was added at the end of the incubation; in the second the hydroxyamino acid was added after incubation.

TABLE VI

ACTIVITY OF CYSTATHIONINE SYNTHETASE ON HYDROXYAMINO ACIDS IN PRESENCE OF HOMOCYSTEINE

Enzyme protein, 0.36 mg; EDTA, $5 \cdot 10^{-4}$ *M*; mercaptoethanol, $1.3 \cdot 10^{-4}$ *M*; 250 μmoles DL-homocysteine.

Hydroxyamino acid added	*Amount added μmoles*	*Cystathionine synthetase activity μmoles homocysteine utilized*
L-serine	200	21
L-threonine	200	0.45
Hydroxy-DL-aspartic acid	100	0.3
L-homoserine	200	0.7

The results indicate that, with the exception of L-serine which is a highly potent substrate for synthetic activity, all of the other amino acids were not utilized by the enzyme.

The results reported in this communication support the previous conclusion[1] that dehydrative deamination of serine and synthesis of cystathionine are functions of a single enzyme protein. Additional evidence has been obtained that pyridoxal phosphate is the coenzyme for the enzyme activities and that the enzyme requires free sulfhydryl groups for activity.

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